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ANNUAL REPORT '96-97

GRANT DAMD17-94-J-4510
Study of Met Tyrosine Kinase in the Pathogenesis of Breast Cancer

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I. Introduction

Breast cancer is the most common malignancy affecting women in the Western World. Epidemiological studies have defined certain factors that may contribute to the risk of breast cancer, the most important one being a family history of the disease (1, 2). Approximately 5-10% of breast cancers are associated with inherited susceptibility with one or more autosomal dominant traits. Linkage at the estrogen receptor on chromosome 6 has also been reported in breast cancer family with a late-onset mode (3). Families with germ-line p53 mutations (Li-Fraumeni Syndrome) often have multiple breast cancers, representing only about 1% of all familial breast cancer cases (4). Molecular analysis of sporadic breast cancer DNAs has revealed loss of heterozygosity on chromosomes 1p, 1q, 3p, 6q, 7q, 8q, 11p, 13q, 15q, 16q, 17p, 17q, and 18q (5). Gene amplification is also frequently observed in the c-myc, HER2/neu, and int-2/PRAD-1 genes of breast tumors (6-8). Furthermore, a significant association between the level of nm23 expression and aggressive tumor behavior has been demonstrated (9). Recently, a breast cancer susceptibility gene (BRAC1) in chromosome 17q13 has been identified (10). These observations suggest that breast cancer, similar to the oncogenesis of other solid tumors, develops through a multistep process involving various genetic alterations. Identification and characterization of these genetic alterations will not only offer the possibility of early diagnosis but also spawn new treatment modalities targeted specifically at the altered phenotype of malignant cells.

Tyrosine kinases have been by far the most important class of mutated cellular genes during malignant transformation. The met oncogene was identified in a N-methyl-N'-nitro-N-nitrosoguanidine(MNNG)-treated human osteosarcoma cell line HOS, using the NIH 3T3 cell transfection system (11-13). The activation of the met oncogene was shown to occur via a chromosomal rearrangement, presumably as a result of the mutagenic effect of MNNG. The rearrangement generates a chimeric gene, fusing an upstream promoter-containing sequence (tpr) from chromosome 1 in front of the carboxyl terminus of the met protooncogene on chromosome 7. The fusion molecule (MW 65 KD) contains the tyrosine kinase domain of the met protooncogene. The tpr sequence consists of a constitutive promoter and an open reading frame coding for a protein with strong sequence homology to nuclear oncoproteins fos, jun, transcription factor CREB, and members of intermediate filament multigene family (14). The common feature

among these molecules is that they contain a leucine zipper which has been shown to be required for dimerization and activation of these proteins (15). Recent data demonstrated that tpr-met oncogene was indeed activated through this leucine zipper interaction, resulting in a constitutively phosphorylated and presumably active state of this tyrosine kinase molecule (16). Overexpression of normal c-met appeares to be sufficient to activate the tyrosine kinase, which may explain the transforming potential of amplified c-met gene in some human tumors (17). The identification and characterization of other forms of c-met has demonstrated that abnormal processing of the extracellular domain of the protein can also result in constitutive activation of c-met (17). Similar to what has been described for other receptor tyrosine kinases (RTK), such as trk and ret proto-oncogenes, mutations affecting the extracellular or transmembrane domain may be the molecular basis for the oncogenic potential of met in some human cancers (18-21).

The proto-oncogene of met encodes a receptor of 190 kDa protein, conposed of two disulfide-linked subunits: an extracellular 50 kDa α-subunit and a transmembrane 145 kDa β-subunit. The receptor is synthesized as a 170 kDa precursor that is glycosylated and cleaved posttranslationally to give the mature heterodimer. The intracellular domain of c-met protein has a structure resembling that of the protein tyrosine kinase (PTK) family. Recent studies have shown the presence of multiple forms of c-met gene products, presumably as a result of alternative splicing (22). The functional significance of these alternative forms has remained largely unknown.

Recently, hepatocyte growth factor (HGF) has been shown to be the ligand for the c-met receptor (23). HGF is also known as hepatopoietin and is identical to scatter factor, which affects the motility, chemotaxis, and invasiveness of epithelial and endothelial cells in culture (24-26). HGF has been shown to be the most potent growth factor for rat and human hepatocytes in primary cultures (27). In addition to its mitogenic effect, it regulates cellular shape as a morphogen and cellular motility as a motogen (24). The pleoitropic effects of HGF-SF on cells suggest a complex interplay of receptor-mediated signal transductions. It is clear from previous studies that HGF appears to induce rather diverse biological effects in various cell types (24). The factors determining the outcome of HGF actions have remained largely unknown. Elucidation of these factors will undoubtedly give us some unique insights into the

molecular mechanisms of growth regulation and malignant transformation.

Cytogenetic studies of transformed cells derived from human malignancies suggested that the mechanism of chromosomal rearrangement resulting in fusion gene products with transforming potential may be a common mechanism of oncogenesis (25). Several examples are the Philadelphia chromosome of chronic myelogenous leukemia, t(14;18) translocation of follicular B cell lymphoma, and t(10;14) translocation of T cell acute lymphoblastic leukemia. Therefore, activation of met proto-oncogene via this mechanism may be more than an in vitro observation in the original mutagenesis experiment described above. In addition, c-met proto-oncogene has been shown to be amplified and overexpressed in a human gastric tumor cell line, although the mechanism of activation is distinct from that of tpr-met activation (26). A recent report described the finding of similar chromosomal rearrangements resulting in tpr-met fusion gene in human gastric tumors (27). This provocative finding is consistent with the observation that nitroso compounds are epidemiologically associated with the occurrence of gastric cancer in humans (28).

Regardless of the oncogenic potential of the activated met protein, its proto-oncogene product likely plays an important role in the growth and differentiation of epithelial cells in various organs. Recent studies have demonstrated a wide tissue distribution of this protein, such as in breast, intestine, stomach, liver, pancreas, kidney, etc., and more interestingly, increased level of met RNA and protein in several carcinoma specimens, particularly in thyroid, gastric and intestinal tumors (29). In contrast, none of 15 primary breast cancers showed expression of met protein, whereas significant met expression was detected in 4 of 4 normal mammary epithelium (29). Another provocative study demonstrated that the c-met locus on chromosome 7 (7q21-22) was deleted in 41% of 245 patients with primary breast cancer (30). In addition, patients with loss of heterozygosity on chromosome 7q21-22 had significantly shorter metastasis-free survival and overall survival. Recent studies on the functions of HGF-met activation have suggested a potential regulatory role for c-met in the morphogenesis of breast tissues (31). These observations, in toto, suggest that this region of chromosome 7, possibly the c-met gene, may be the site of a breast tumor or metastasis suppressor gene. The concept of a RTK as a tumor suppressor gene has been substantiated by a recent report demonstrating that the RET tyrosine kinase gene appears to

code for the tumor susceptibility gene in Multiple Endocrine Neoplasia Type II Syndrome (MEN II) (32).

In this grant, we plan to search for genetic abnormalities affecting the c-met gene at the DNA, RNA, and protein levels in human breast cancer cell lines and tumor specimens (Specific Aim A), and study the key cellular targets of c-met gene product in mammary epithelial cells and define the functional effects of mutated met proteins through exploring a innovative technology to identify and characterize interacting proteins (Specific Aim B). We are applying the two-hybrid system in yeast in order to clone cellular genes whose protein products interact specifically with the met protein. As Specific Aim C, we plan to develop animals models to examine the oncogenic potentials of met oncogene and to assess the role of met proto-oncogene in the growth and development of breast tissues.

II. Body

We are applying a variety of molecular and cellular approaches to study the role of c-met in the development of breast cancer. We have generated significant amounts of preliminary data regarding each of the specific aims. There has been a change of personnel in this grant over the last year. The original Principal Investigator, Dr. Liang has departed MGH and his collaborator Dr. Timothy Wang has assumed the role of PI since October of 1996. In addition, many research personnel included in the original grant application has also turned over. Because of this major change in personnel, we have not made substantial progress of the proposed studies over the last year. For Specific Aim A, we have examined the status of c-met gene and its product(s) in several breast cancer cell lines and have observed that the expression of met gene in these lines appeared to be absent or altered with aberrant forms of gene product (Table 1). For Specific Aim B, we have initiated our effort to clone cellular target(s) of c-met in order to understand the signal transduction pathway leading to malignant transformation of mammary epithelium using the two-hybrid system. This innovative technology has been developed and used extensively to clone and identify cellular factors interacting with specific proteins of interest. The system has been described in detail (33, 34).

In brief, two different DNA constructs capable of replicating in yeast are prepared (Fig. 1). In the first construct, the gene coding for the protein of interest is fused to a gene encoding a

known DNA-binding polypeptide that dose not activate transcription by itself, such as the DNA binding domain of GAL4 (aa 1-147) or LexA (aa 1-202). The second construct containing a gene coding for an activation domain, such as that of GAL4 (aa 768-881) or other transcription activator, is fused to a cDNA library of interest, as the "prey." The second construct, by itself, does not activate transcription, since it contains no DNA binding domain. The reporter construct contains the cognate DNA binding sequence of the DNA binding domain upstream of a gene that produces an protein with enzymatic activity, such as b-galactosidase (b-gal), or an auxotrophic marker, such as Leu-2, which is required for leucine synthesis. The interaction of two proteins will bring the DNA binding and activation domains together to activate the transcription of the reporter gene, resulting in selection of the yeast clone containing the candidate gene whose gene product interacts with the protein of interest. The system we have adopted was provided generously by Dr. Roger Brent of the Dept of Molecular Biology at MGH. We have identified several candidate genes whose gene products interact specifically with the intracellular domain of c-met in the yeast two-hybrid system (Table 2). A recent publication using the same approach has demonstrated that the protein Gab1 which is a signal transduction adaptor interacts with the cytoplasmic domain of c-met (35). Further characterization of other clones may lead to a better understanding of how mutated c-met induces breast cancers.

Finally for Specific Aim C, we have generated four transgenic founder lines expressing the met oncogene (tpr-met). Three lines positive for tpr-met transgene were successfully breeded and established. The results of these studies have been published in the Journal of Clinical Investigation (see attached manuscript). In summary, A F1 female animal from the MTM1 line, after breeding for 6 months, developed a mammary cancer. Nine additional F1 females from this line, 8 from line MTM2, and 8 from line MTM3 were set up for forced continuous breeding (see attached manuscript). Most of the multiparous mice had mammary hyperplastic alveolar nodules (HAN) (see attached manuscript) and several of them also had foci of microscopic carcinoma on whole mount examination. Seven primary mammary tumors developed in 6 of the MTM1 animals. In the MTM2 line, 7 independent mammary tumors developed in three female animals. In the third line (MTM3), 3 mammary tumors were observed in two animals. The mammary tumors had one of three patterns, scirrhous, papillary or nodular , many of them resembling human mammary tumors. The nuclei were intermediate in size with

delicate clumped chromatin. Histological patterns of these types are not seen in spontaneous murine tumors.

Additional tumors were also found in animals from all three lines. Single animals from MTM1 and MTM3 lines developed diffuse lymphoblastic lymphomas involving the mammary gland and lymph nodes at 6-9 months of age. One MTM3 mouse developed a thymic lymphoma. Spindle cell sarcomas were also observed in MTM2 and MTM1 mice. An unusual orbital giant cell osteosarcoma was noted in another MTM1 animal at 18 months of age. In addition to continuing our analyses of these lines, we had hope to extend our efforts to study the biological effects of inactivated met gene in transgenic mice with germ-line disruption of the c-met gene in our original proposal. Unfortunately, the same study has been published recently in Science (36). Since the homozygous knock-out is embryonically lethal, it is diffcult to analyze the effect of this genotype on mammary development. We are currently contemplating an alternative approach to address this issue. Techniques are now available for tissue-specific conditional knock-out using the Cre-loxP system (37, 38). In collaboration with Dr. Brian Sauer of NIH, who has pioneered this system, we are generating constructs to pursue this avenue of research.

In addtion, we are also developing another transgenic approach to study the effect of inducible expression of met oncogene on malignant transformation of mammary glands and other tissues. This approach takes advantage of the tight regulation of gene expression in the bacterial tetracycline operon by tetracylcine (39). The tetracycline repressor (tetR) negatively regulates expression of sequences under the control of tet operon (tetO) sequences. In the presence of TCN, tetR does not bind to the operator sequences and transcription is allowed. This has been adapted to facilitate tetR-mediated transcriptional activation. The tet repressor is fused with the activating domain of HSV virion protein 16 (VP16) as the tetR-VP16 along with a neomycin resistance gene (pUHD15.1neo), thus generating a TCN-controlled transactivator (tTA). The gene of interest is inserted downstream of a tTA-responsive tet operon and CMV minimal promoter sequence (derived from plasmid pUHC13-3). In the presence of TCN, tTA does not bind to the tetO sequences and only basal levels of transcription occur (Fig. 2). Recently, the tetracycline inducible system has been used to modulate the expression of genes in mammalian cells in vitro and in vivo, when placed under the control of a tissue-specific promoter (40, 41).

The tpr-met cDNA is inserted 3' to the tet operator sequence linked to a thymidine kinase minimal promoter, thus allowing

control of tpr-met expression by tetracycline (TCN) when a second transgene expressing the tetracycline controlled transactivator is present. This construct will be used for transgenic injection. We have also generated 6 trangenic lines (three under the albumin protomer and the other three under the mouse urinary promoter) that express the tetR-VP16 in the liver. Our collaborator, Dr. Lothar Hennighausen, who has generated transgenic lines containing the tetR-VP16 under the MMTV promoter which should allow inducible mammary gland expression (41, 42), has agreed to provide us with these lines. If we can achieve inducible expression of tpr-met in mammary glands, we can study the oncogenic process of tpr-met-induced mammary carcinoma under carefully controlled conditions.

As an overall goal of the proposal, by exploring these three distinct but complementary approaches, we hope to gain important insights into the multistep pathogenesis of breast cancer.

III. Conclusions

From our preliminary studies on genetic alterations involving the c-met gene, we observed an association with tumorigenicity of cell lines and aberrant expression of c-met (Specific Aim A). This observation is consistent with our hypothesis that dysregulated synthesis of c-met plays a role in breast carcinogenesis. Identification and characterization of signal transduction pathway of c-met in mammary tissues will likely provide us with important information regarding the molecular mechanism of met-induced mammary carcinogenesis (Specific Aim B).

We have performed extensive studies in the transgenic model of met (Specific Aim C) and these studies have already been published (attached manuscript). The pattern and occurrence of mammary hyperplasia and tumors in the tpr-met transgenic strains support strongly the conclusion that these lesions are a direct effect of the transgene. Hyperplasia and tumors developed in all three independent transgenic lines (MTM1, MTM2, and MTM3). In addition, no spontaneous mammary tumors developed in any of the nontransgenic littermates maintained in parallel. This observation is consistent with other investigator's experiences with the FVB strain. Furthermore, occurrence of multiple independent mammary tumors, as observed in some of our animals, is rare in spontaneous breast tumors of normal mice. The use of a cellular promoter (metallothionein) rather than the MMTV promoter to drive the tpr-met transgene further speaks for the uniqueness of this animal model in the study of mammary carcinogenesis. It is interesting to

note that only breast tumors expressed high-level of the tpr-met transcript and protein, whereas normal tissues including mammary epithelium expressed very little. Perhaps, during physiological hyperplasia of mammary glands as a result of pregnancy, cells expressing higher levels of the oncoprotein were selected and clonally expanded to eventually form foci of mammary malignancy. This observation suggests that mammary adenocarcinoma developed in this transgenic model as a direct effect of high-level expression of tpr-met. Development of conditional and/or inducible expression of tpr-met in transgenic mice will afford great opportunities to study the subtle and intricate aspects of mammary carcinogenesis in this model.

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V. Appendix

Table 1: Altered c-met Expression in Breast Cell Lines.

Cell Line*	Tumorigenic**	DNA***	RNA#	Protein
75N	-	+	+	ND
70N	-	+	+	+
81NN	-	+	+	ND
21NT	-	+	+	+
21PT	-	+	+	ND
18-2-1	-	+	+	ND
1436N1	-	+	+	ND
HBL-100	-	+	+	+
Hs578Bst	-	+	+	+
MDA-MB-157	+	+	-	ND
MDA-MB-231	+	+	-	ND
MDA-MB-361	+	+	-	ND
MDA-MB-435	+	+	+	+
MDA-MB-436	+	+	+	ND
MDA-MB-468	+	+	-	ND
BT-474	+	+	-	ND
BT-549	+	+	-	-
MCF7	+	+	-	-
MCF10A	+	+	+	ND
T-47D	+	+	-	ND
ZR-75-1	+	+	-	-
ZR-75-3	+	+	-	-
DU4475	+	+	-	-
Hs578T	+	+	+	-
SK-BR3	+	+	+	-

* The primary mammary cells 76N and 70N, and immortalized breast cell lines 21-NT, 21-PT, 18-2-1, and 1436N1 are from Ruth Sager; the remaining cell lines are obtained thru ATCC.

* * "Nontumorigenic" is defined as inability to form tumor in nude mouse.

* * * Absence of gross gene rearrangement by restriction and Southern blot analysis is defined as "+".

\# presence or absence of met mRNA was indicated by + or -.

ND: Not done

Table 2. Interaction of Intracellular Domain of c-met with Candidate cDNA Clones

Yeast Plasmid Construct	β-gal Activity (U) Glucose	Galactose
LexA-MetCD + JG4-5	10	12
LexA-MetCD + JG4-5-MAP51	25	1939
LexA-MetCD + JG4-5-MAP142	26	2789
LexA-MetCD + JG4-5-MAP191	31	1897
LexA-MetCD + JG4-5-MAP282	27	2192
LexA + JG4-5-MAP51	17	19
LexA + JG4-5-MAP142	15	21
LexA + JG4-5-MAP191	11	14
LexA + JG4-5-PMAP282	13	16

Figure 1.

The Yeast Two-Hybrid System

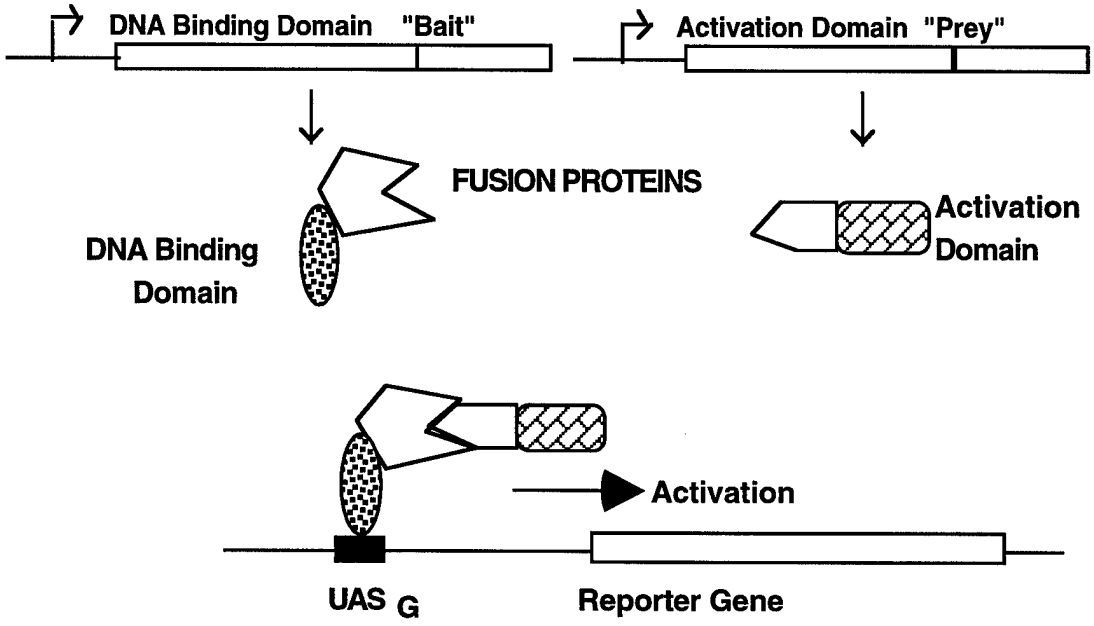

Figure 2. The Tetracycline Transactivating System.